**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68

A2

(11) International Publication Number: WO 91/09973

(43) International Publication Date: 11 July 1991 (11.07.91)

(21) International Application Number: PCT/GB90/02031

(22) International Filing Date: 28 December 1990 (28.12.90)

(30) Priority data:
8929293.2 29 December 1989 (29.12.89) GB

(71) Applicant *(for all designated States except US)*: 3i RESEARCH EXPLOITATION LIMITED [GB/GB]; The Gate House, 2 Park Street, Windsor, Berkshire SL4 1LU (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : TABAQCHALI, Soad [IQ/GB]; 9 Kent Terrace, London NW1 4RP (GB). CLAYTON, Christopher, Leeson [GB/GB]; 11 School Lane, Aston, Stevenage, Hertfordshire SG2 7HA (GB). WREN, Brendan, William [GB/GB]; 5 Leaside Walk, East Side, Ware, Hertfordshire SG12 9AB (GB).

(74) Agents: LAMBERT, Hugh, Richmond et al.; D. Young & Co., 10 Staple Inn, London WC1V 7RD (GB).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: C. DIFFICILE SPECIFIC OLIGONUCLEOTIDES

(57) Abstract

Oligonucleotides having substantially specific binding affinity towards *C. difficile* DNA are disclosed, especially oligonucleotides specific to the *C. difficile* A-toxin gene. Such oligonucleotides are useful as DNA probes and PCR primers in the detection of *C. difficile* in human clinical samples, e.g. facal samples.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| **AT** | Austria | **ES** | Spain | **MG** | Madagascar |
| **AU** | Australia | **FI** | Finland | **ML** | Mali |
| **BB** | Barbados | **FR** | France | **MN** | Mongolia |
| **BE** | Belgium | **GA** | Gabon | **MR** | Mauritania |
| **BF** | Burkina Faso | **GB** | United Kingdom | **MW** | Malawi |
| **BG** | Bulgaria | **GN** | Guinea | **NL** | Netherlands |
| **BJ** | Benin | **GR** | Greece | **NO** | Norway |
| **BR** | Brazil | **HU** | Hungary | **PL** | Poland |
| **CA** | Canada | **IT** | Italy | **RO** | Romania |
| **CF** | Central African Republic | **JP** | Japan | **SD** | Sudan |
| **CG** | Congo | **KP** | Democratic People's Republic of Korea | **SE** | Sweden |
| **CH** | Switzerland | | | **SN** | Senegal |
| **CI** | Côte d'Ivoire | **KR** | Republic of Korea | **SU** | Soviet Union |
| **CM** | Cameroon | **LI** | Liechtenstein | **TD** | Chad |
| **CS** | Czechoslovakia | **LK** | Sri Lanka | **TG** | Togo |
| **DE** | Germany | **LU** | Luxembourg | **US** | United States of America |
| **DK** | Denmark | **MC** | Monaco | | |

# C. DIFFICILE SPECIFIC OLIGONUCLEOTIDES

This invention relates to oligonucleotides specific to bacteria of the species Clostridium difficile and useful as C. difficile DNA probes or in the amplification of C. difficile DNA.

Clostridium difficile, a Gram positive anaerobic spore-forming organism, is recognised as the major cause of pseudomembranous colitis and is implicated in antibiotic-associated colitis and diarrhoea. The pathogenicity of the organism is related to the production of an enterotoxin, toxin A, and a potent cytotoxin, toxin B. The diagnosis of C. difficile-associated disease depends on the isolation and identification of the organism and/or the demonstration of toxins in faecal specimens of patients and mono-specific antibodies for that purpose are disclosed in EP-A-0153519 and EP-A-0154064. Such procedures are time consuming, and a more rapid diagnosis is essential to enable the initiation of prompt therapy.

The present invention aims to avoid these difficulties by providing oligonucleotides specific to C. difficile DNA and is a development of the work reported in J. Clin. Microbiol. 24, 3, pp 384-387 (Sept. 1986). In that paper, the results of immunoblot studies on various strains of C. difficile were reported and various immunogenic proteins were identified common to all nine C. difficile strains tested. In particular, three common antigens were potentially identified with molecular weights in the range 50 to 70 kDa.

Following on from those studies, further antigens have now been identified common to all C. difficile strains tested, and the genes encoding those antigens have been identified, cloned and, in one case, sequenced giving rise to the present invention, namely the construction, for the first time, of DNA probes and oligonucleotide primers specific to C. difficile DNA.

More specifically, in accordance with a first aspect of the present invention a 1947 kb DNA fragment encoding part of the A toxin gene of C. difficile has been identified, cloned and sequenced, the sequence being as set out in the Appendix I hereto as SEQ ID No.1 and which is to be taken as forming part of the present specification.

Analysing that 1947 kb sequence in more detail, a continuous open reading frame is found containing four distinct groups of repeat nucleotide sequence with 88 to 100% identity within each group. These groups, identified herein as A, B, C, D (A = 81bp; B, C, D = 63bp) appear in the following arrangement:

ABCCCDABCDDABCCCDABCCDABCDABC

where

A = 5' ATA GGG GTG TTT AAA GGA CCT AAA GGA TTT GAA TAT TTT GCA
Ile Gly Val Phe Lys Gly Pro Lys Gly Phe Glu Tyr Phe Ala

CCT GCT AAT ACT TAT AAT AAT AAC ATA GAA GGT CAG GCT 3'
Pro Ala Asn Thr Tyr Asn Asn Asn Ile Glu Gly Gln Ala

B = 5' ATA GTT TAT CAA AGT AAA TTC TTA ACT TTG AAT
Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn

GGT AAA AAA TAT TAC TTT GAT AAT AAC TCA 3'
Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser

C = 5' GAA GCA GCT ACT GGA TGG CAA ACT ATT GAT GGT
Glu Ala Ala Tyr Gly Trp Gln Tyr Ile Asp Gly

AAA AAA TAT TAC TTT AAT ACT AAC ACT GCT 3'
Lys Lys Tyr Tyr Phe Asn Tyr Asn Tyr Ala

and

D = 5' ATA GCT TCA ACT GGT TAT ACA ATT ATT AAT GTT
Ile Ala Ser Thr Gly Tyr Thr Ile Ile Asn Gly

AAA CAT TTT TAT TTT AAT ACT GAT GGT ATT 3'
Lys His Phe Tyr Phe Asn Thr Asp Gly Ile

The repeating sequence of the A, B, C and D blocks given above account for 1935 bp of the 1947 bp fragment. The remaining 12 are accounted for two linking areas each of 6bp which occur between the A and D groups underlined.

Utilising that information oligonucleotides and DNA probes capable of hybridising to the C. difficile A toxin gene and potentially useful in the early detection of C. difficile infection may now be constructed. The probes may range in size from virtually the complete 1947 nucleotide sequence given in the appendix hereto, SEQ ID No 1 down to sequences of a more appropriate length for oligonucleotide synthesis, for example, sequence of from 15 to 300 nucleotides, more usually of from 15 to 50 nucleotides and especially from 15 to 30. The final selection of a particular oligonucleotide, or pair of oligonucleotides will depend upon a number of factors, well understood in the art, and including amongst others the stringency requirements, i.e. the ability or otherwise of the probe to tolerate local mismatching with the complementary sequence in the target DNA. Obviously the longer the probe the better the ability to withstand local mismatching without adversely affecting the hybridisation of the oligonucleotide to the target DNA. However, the length of the oligonucleotide always has to be balanced against other factors such as ease of synthesis. The factors affecting that choice are, however, well recognised and well within the capabilities of the person skilled in the art.

Also, as the person skilled in the art will recognise, references herein to particular oligonucleotides and sequences in single stranded form and including the complete 1947 bp sequence, and which are written, as is required, reading from left to right, i.e. from the 5' terminus to the 3' terminus, automatically include the complementary sequence. Not only that, but oligonucleotide sequences given herein as DNA sequences can equally well be constructed as RNA sequences with uracil (U) replacing thymine (T).

Whilst, as indicated, the generality of the present invention extends to DNA (and RNA) probes complementary to any sequence of nucleotide bases to be found in the 1947 bp sequence set out hereinafter, certain sequences and pairs of sequences can be identified as being particularly preferred. For example, sequences selected from the repeat sequences already given are especially preferred as being complementary to two spaced but identical sections of the C. difficile A toxin gene. One such sequence is the 25 unit oligonucleotide:

BW71: 5' AAACTATTGA TGGTAAAAAA TATTA 3'

(BW71 is simply an identification code) which sequence is to be found in the Toxin A Repeat C sequence given above.

A more particular aspect of the present invention relates to selected pairs of C. difficile oligonucleotide probes which can be used to amplify the C. difficile A toxin gene or sections thereof by polymerase chain reaction (PCR) technology and using selected pairs of oligonucleotides as primers.

In recent years PCR has developed into a major tool in DNA probe technology enabling highly sensitive detection of minute amounts of genetic material present in a sample by first amplifying the gene or gene fragment by PCR to replicate a multiplicity of identical gene or gene fragments. Not only does that enable better detection of that gene or gene fragment, but it provides a substantial reservoir of genetic material for subsequent research and analysis, and for subsequent cloning and expression in a suitable vector. However, PCR presupposes at least some knowledge of the nucleotide sequence in the gene or gene fragment to be amplified, in view of the need to provide an oligonucleotide primer for the PCR, which primer hybridizes to the complementary sequence in the gene or gene fragment to be amplified and which initiates the PCR replication of the gene or gene fragment from that point. The use of a pair of primers which hybridize to the target DNA at different spaced apart locations enable precise lengths of the target gene or gene fragment to be amplified.

Once again, it is theoretically possible to use any pair of oligonucleotides hybridizable at different points of the C. difficile A toxin gene as primers for the PCR amplification and subsequent detection of that gene, and consequently the detection of C. difficile in a sample, e.g. a clinical sample. However, it is preferable to use as primers for the PCR oligonucleotide which hybridise to spaced locations on one of the repeat sequences now identified as appearing in the C. difficile A toxin gene. Thus, in the amplification of the gene by PCR replication of the repeat sequence occurs in parallel at each location of that sequence in the gene, thus substantially increasing the replication rate, and increasing not only the sensitivity of the C. difficile detection, but also dramatically reducing the time required to carry out a diagnosis of C. difficile infection in a patient. In tests carried out by the present inventors, positive diagnoses of C. difficile infection have been confirmed by PCR within the space of five hours.

Based on the repeat sequences given above, various oligonucleotide pairings can be suggested of various different lengths and comprising a sequence of nucleotides complementary to the sequences at the 5'- and 3'- ends of each repeat sequence. Especially preferred are pairs of oligonucleotides complementary to the 5' and 3' termini of the tandem C repeat sequence. Two particular pairings which have been synthesized and used in accordance with this invention are

BW69: 5' GAAGCAGCTA CTGGATGGCA 3'
BW70: 5' AGCAGTGTTA GTATTAAAGT 3'

complementary to the opposite ends of the C. difficile A toxin gene repeat sequence C,

and

BW65: 5' ATAGGGGTGT TTAAAGGACC T 3'
BW66: 5' AGCCTGACCT TCTATGTTAT 3'

complementary to the opposite ends of the C. difficile A toxin gene repeat sequence A.

In general terms, the preferential pairings for PCR amplification of the C. difficile A toxin gene fragments may be identified as oligonucleotide sequences of from 15 - 25 nucleotides complementary to the 15 to 25 nucleotide sequences at the 5' and 3' ends of the A, B, C and D repeat sequences identified above, and especially of the A and C sequences.

In accordance with a second aspect of the present invention a 31 kDa antigen has been identified as common to all C. difficile strains tested, and has been found to be encoded by a 1.8 kb HindIII digestion fragment of C. difficile DNA. Thus, in accordance with a second aspect of the present invention, C. difficile probes are provided derived from or complementary to 1.8 kb HindIII digestion fragment of C. difficile or to individual sequences of that fragment and comprising oligonucleotides again ranging in size from 15 units to substantially the complete sequence, but more usually ranging in size from 15 to 300 units, most usually 15 to 50 units, more preferably 15 to 30 units.

Although the correct reading frame has yet to be established, the complete sequence of the 1.8 kb HindIII digestion fragment encoding the 31 kDa C. difficile antigen is as set out in Appendix II as SEQ ID No.2 and which again is to be taken as form a part of the present specification.

Particular oligonucleotide probes complementary to selected sequences of the 1.8 kb HindIII fragment are identified as follows, the numbers in parentheses are simply identification codes.

| Probe | Hind III Fragment Sequence nt. numbers |
|---|---|
| (028) 5'AAAAATGCTC AGCTTGCAG 3' | 318 - 336 |
| (031) 5'AAAAATTTAA CTAAGTT 3' | 734 - 750 |
| (029) 5'AACAATTTTA TTTAAAC 3' | complementary to 1460-1476 |
| (032) 5'TTTTGATGTT AACTGACC 3' | complementary to 1058-1075 |
| (037) 5'GTTACTTTTG ATACAGTA 3' | 1076 - 1093 |

In a broad aspect therefore, the present invention provides C. difficile specific oligonucleotides comprising oligonucleotides of at least 15 nucleotides complementary to, and capable of hybridisation with, any sequence of 15 or more nucleotide bases of the DNA sequences hereinbefore set forth and identified respectively as the 1.8 kb HindIII DNA fragment of C. difficile and the 1.974 kb A Toxin DNA fragment, said probes having a length upto and including the complete sequence.

Within that broad class, two broad sub-categories can be identified as constituting or including the preferred C. difficile specific oligonucleotides according to this invention. The first sub-category is constituted by sub-restriction fragments of either complete sequence, and as such sub-restriction fragments there may be especially identified the following:

An Eco RI/PstI 1448 bp sub-restriction fragment of the Toxin A gene.

Such restriction fragments may, of course, be obtained in known manner, for example by digestion of C. difficile genomic DNA with the appropriate restriction enzyme or enzymes and subsequent separation by gel electrophoresis, chromatography or in any other suitable manner known for the separation of individual DNA restriction fragments according to size.

The second principal sub-category of DNA probes according to the invention are synthetic oligonucleotide sequences, e.g. those already mentioned. Such oligonucleotide sequences are readily assembled using known oligonucleotide synthesis techniques.

Utilising selected complementary pairs of the above oligonucleotides as primers, polymerase chain reactions (PCR) have been carried out according to manufacturers specifications (Cetus Ltd.) to amplify known fragments of C. difficile DNA, both purified total C. difficile DNA and crude C. difficile DNA released from C. difficile bacterial cells. In these experiments:

oligonucleotides 031 and 032 have been shown to amplify a 0.303 kb fragment of C. difficile DNA;

oligonucleotides 028 and 029 have been shown to amplify a 1.2 kb fragment of C. difficile DNA;

oligonucleotides 031 and 029 have been shown to amplify a 0.7 kb fragment of C. difficile DNA;

oligonucleotides 037 and 029 have been shown to amplify a 0.35 kb fragment;

oligonucleotides 029 and 032 have been shown to amplify a 0.8 kb fragment of C. difficile DNA; and

Although PCR amplification appears to be the presently preferred method of C. difficile detection using the C. difficile specific oligonucleotides of this invention, other detection procedures are available and are well known in the art. To this end the C. difficile specific oligonucleotides of this invention may be provided with a variety of different labels: radioactive, fluorescent or enzyme, to provide a variety of DNA probes specific to C. difficile DNA and all permitting the detection of any hybridised (bound) DNA probe to the unidentified DNA sample under investigation. Alternatively, of course, for sandwich hybridisation techniques the DNA probes of this invention may be immobilised in any known appropriate fashion, e.g. by covalent bonding to a variety of different solid substrates, both particulate,

e.g. glass, Sephadex, Sephacryl beads etc., and continuous surface substrates. The techniques of DNA labelling and immobilization are well known in the art, as are methods for the detection of microorganisms, in general, utilising DNA probes and which will be equally suitable in accordance with the present invention for the specific detection of C. difficile. Such procedures and methods are not part of the present invention as such and need not be further described here.

However, it is to be understood that references herein to an oligonucleotide for use as DNA (or RNA) probe in accordance with this invention are to be taken as including also labelled, tailed and immobilised versions thereof as well as the oligonucleotide sequence per se, it also being understood that oligonucleotide lengths given herein do not include additional non-complementary sequences, such as poly(DA) or poly(DT) tails added for a specific purpose such as immobilising the probe on a poly(DT) or poly(DA) coated surface.

The C. difficile specific oligonucleotides covered by this invention include both single- and double-stranded versions, it being understood that in any subsequent hybridisation procedures such as the detection of C. difficile in clinical, faecal or other samples, such double-stranded oligonucleotide will require denaturing to provide the probes in single-stranded form.

Also included within the scope of this invention are a method of detecting the presence of C. difficile in a sample, for example a faecal sample, which comprises treating the sample to release the DNA or mRNA and detecting the presence of C. difficile DNA or mRNA using a C. difficile specific oligonucleotide according to the invention, and especially a pair of such oligonucleotides as primers for a PCR reaction and diagnostic kits for the diagnosis of C. difficile infection in a patient comprising a C. difficile specific oligonucleotide according to the invention, and especially PCR kits containing a pair of such oligonucleotides for use as primers in the PCR reaction.

The invention is illustrated by the following Examples:

Example 1

PCR amplification of C. difficile A toxin gene using oligonucleotides BW69 and BW70

Sample Preparation

Nine standard C. difficile strains, labelled A to E and W to Z were grown anaerobically at 37°C for 36 hours on selective media (cycloserine, cefoxitin and fructose sugar). Strains B, D, E, W, X and Z were specifically selected as being known to be toxigenic. Strains A, C and Y selected as controls were known to be non-toxigenic.

Single colonies of each strain were scraped with an inoculating loop into a 1.5 ml polypropylene tube containing 200 sterile water and boiled for 10 minutes. The samples were then centrifuged for 5 minutes at 14000 x g to obtain a supernatant C. difficile cell extract.

Oligonucleotide primer synthesis

Oligonucleotides BW69 and BW70 (see above) were synthesised on an Applied Biosystems synthesiser using the automated phosphoramidite coupling method.

PCR

Two microlitres of each C. difficile extract were added to a 100 reaction volume with 10mM tris-HCl (pH 8.3), 0.01% gelatin, 200 of each of the four deoxyribonucleotides: A, C, T, G, 100pM of each of the two primers and 2.5U Taq polymerase (Perkin-Elmer Cetus, California). The reaction mixtures were overlain with paraffin oil (100 ) placed in a thermal cycler (Hybaid Ltd.) and amplified for 30 cycles. A rapid two step cycle of 94°C and 46°C for 30 seconds each was used except that the first 94°C step was extended to 3 minutes to ensure denaturation of the initial sample.

Product Separation

20 aliquots of the amplified samples were electrophoresed in a horizontal 4% Nusieve GTG agarose gel (ICN Biochemicals Ltd.) containing 0.5 ethidium bromide per ml, and the bands visualised under UV light.

In a parallel series of experiments, the cultured C. difficile strain W was serially diluted to provide samples containing 300, 30 and 3 bacteria (as well as $10^7$ other anaerobic enteric bacteria from the original faecal sample) which were likewise subjected to PCR with primers BW69 and BW70 (samples 1, 2 and 3).

Results

The results are shown in Figure 1 of the accompanying drawings which shows the band separation obtained with each of the samples A to E, W to Z and 1, 2 and 3. Lane M is that of a parallel DNA size marker giving bands at 67 80 110 147 190 242 and 267bp.

Lanes B, D, E, W, X, Z, 1 and 2 (toxigenic C. difficile strains) each show a characteristic five bands at 63 126 189 252 and 315 bp, which are not present in samples from the non-toxigenic strains (A, C and T), but which are apparent with samples containing as few as 300 or even 30 bacteria.

These results have been confirmed on a total of 45 C. difficile strains by PCR amplification using the primer pair BW69 and BW70. Of the 45 strains tasted, all 33 strains known to be toxigenic proved positive, whilst the 12 non-toxigenic strain proved negative.

Of 16 strains from 13 different species of Clostridium other than C. difficile, only three, all C. sordelii, have given positive results.

These results are tabulated in Table 1 and clearly show the substantial specificity of the present oligonucleotides towards toxigenic C. difficile, and the fact that positive diagnosis can be made on the basis of as few as 30 bacteria establishes its sensitivity. One of the reasons for this degree of sensitivity is conjectured to be the specific selection of a pair of primers complementary to the 5' and 3' ends of the C. difficile A toxin gene repeat sequence C. Virtually equivalent results can, however, be predicted for similar pairs of primers complementary to the 5' and 3' ends of the repeat sequences A, B and D.

Example 2

PCR amplification of C. difficile DNA using oligonucleotide probes 028 and 029

Oligonucleotides 028 and 029 were synthesised as before on an Applied Biosystems synthesiser.

The two primers were then used at a concentration of 1 M to amplify C. difficile DNA obtained by boiling C. difficile cells in 50 of $H_2O$ for 10 minutes. The DNA was amplified using a cycle profile of 94°C 1 min., 31°C 1 min., and 72°C 3 min. After the last cycle the polymerisation step was extended from 3 to 10 minutes. Twenty six cycles of amplification were performed in total. The reactions were carried out in 100 volumes, 20 samples were run on agarose gels and amplified DNA detected by ethidium bromide staining and comparison with mol. wt. standards. By this method C. difficile DNA has been amplified from cell population as low as $10^2$ C. difficile cells, thus indicating the extreme sensitivity of C. difficile specific oligonucleotides according to this invention. Simultaneous controls with other Clostridia spp. have failed to produce any evidence of amplification, indicating the specificity of probes according to the present invention to C. difficile.

Confirmation that the amplified fragments are C. difficile DNA is obtained both by hybridisation with internal oligonucleotide probes and by using two internal primers for PCR after initial amplification, e.g. by using probes 028 and 029, to give amplified fragments of 1.2 kb, and then using 5% of this reaction product to perform further PCR with probes 037 and 029 to give a 0.35 kb fragment.

Example 3

PCR amplification of C. difficile DNA using primers BW65 and BW66.

Following the same protocol as in Example 1, DNA was amplified from toxigenic and non-toxigenic C. difficile strains using primers BW65 and BW66.

The results are shown in Figure 2. Lanes B D E W X and Z show the PCR amplified products from pure colonies of toxigenic C. difficile and Lanes A C and Y the non-toxigenic strains. Lanes 1, 2 and 3 show serial dilutions of G toxigenic C. difficile strain W at 300 30 and 3 bacteria mixed with $10^7$ other anerobic enteric bacteria. Lane M is a molecular weight size marker showing DNA lanes at 67 80 110 147 190 242 and 267 bp. (BshI and MspI digests of plasmid pHC 314).

The results obtained in Examples 1 and 3 are summarised in Table I below.

Table 1. Summary of PCR experiments with various clostridial strains using either pair of primers BW 65/66 or BW 69/70.

| | No. tested | PCR positive | PCR negative |
|---|---|---|---|
| Toxigenic C. difficile | 33 | 33 | 0 |
| Non-Toxigenic C. difficile | 12 | 0 | 12 |
| Other Clostridial species | 16 | 3 | 13 |

These results clearly establish the specifity and sensitivity of the preferred oligonucleotides of the present invention i.e. those complementary to the terminal sequences of the A and C A-toxin repeat sequences, and indicate their utility as a tool in the detection and diagnosis of infections by C. difficile.

APPENDIX 1.

SEQ ID No.1

| | |
|---|---|
| Sequence Type: | Nucleotide |
| Sequence Length: | 1947 bp |
| Strandedness: | Double |
| Topology: | Linear |
| Molecule Type: | Genomic DNA |
| Original Source: | C. difficile |
| Immediate Experimental Source: | Bacteriophage λEMBL3 C. difficile gene library screening and subcloning into E. coli |
| Features: | Not yet fully established. |
| Properties: | C. difficile A Toxin gene (fragment) |

5'ATAGGGGTGTTTAAAGGACCTAAAGGATTTGAATATTTTGCACCTGCTAATACTTATAATAATAACATAGAAGGTCAGGC
TATAGTTTATCAAAGTAAATTCTTAACTTTGAATGGTAAAAAATATTACTTTGATAATAACTCAAAAGCAGTTACCGGAT
GGCAAACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGATGGCAAACTATTGATGGT
AAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGATGGCAAACTATTGATGGTAAAAAATATTACTTTAA
TACTAACACTGCTATAGCTTCAACTGGTTATACAATTATTAATGGTAAACATTTTTATTTTAATACTGATGGTATTATGC
AGATAGGAGTGTTTAAAGGACCTAATGGATTTGAATATTTTGCACCTGCTAATACGGATGCTAACAACATAGAAGGTCAA
GCTATACTTTACCAAAGTGAATTCTTAACTTTGAATGGTAAAAAATATTATTTTGATAATAACTCAAAAGCAGTTACCGG
ATGGCAAACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTATAGCTTCAACTGGTTATACTATTATTAATG
GAAAACATTTTTATTTAAATACTGATGGTATTATAGCTTCAACTGGTTATACAATTATTAATGGTAACCATTTTTATTTT
AATACTGATGGTATTATGAAAATAGGGGTTTTTAAAGGACCTAATGGATTTGAATATTTTGCACCTGCTAATACTGATCA
TAATAATATAGAAGGTCAGGCTATAGTTTATCAAAGTAAATTCTTAACTTTGAATGGTAAAAAATATTATTTTGATAATA
ATTCAAAAGCAGTTACTGGATGGCAAACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACT
GGATGGCAAACTATTGATGGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGATGGCAAACTATTGA
TGGTAAAAAATATTACTTTAATACTAACACTGCTATAGCTTCAACTGGTTATACAATTATTAATGGTAAACATTTTTATT
TTAATACTGATGGTATTATCGGAGTGTTTAAAGGACCTAATGGATTTGAATATTTTGCACCTGCTAATACTGATGCTAAT
AACATAGAAGGTCAAGCTATAGTTTATCAAAGTAAATTTTTAACTTTGAATGGTAAAAAATATTACTTTGATAATAACTC
AAAAGCAGTTACTGGATGGCATACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGAT
GGCAAACTATTGATGGTAAAAAATATTACTTTAATACTAACACTGCTATAGCTTCAACTGGTTATACAATTAATAATGGA
AAACATTTTTATTTTAATACTGATGGTATTATAGGAGTGTTTAAAGGACCTAATGGATTTGAATATTTTGCACCTGCTAA
TACGGATGCTAACAACATAGAAGGTCAAGCTATAGTTTATCAAAGTAAATTCTTAACTTTGAATGGTAAAAAATATTATT
TTGATAATAACTCAAATGCTGTTACCGGATGTCAAACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTATA
GCTTCAACTGGTTATACAATTATTAATGGTAAACATTTTTATTTTAATACTGATGGTATTATAGGGGTGTTTAAAGGTCC
TAATGGATTTGAATATTTTGCACCTGCTAATACTTATGATAATAACATAGAAGGTCAGGCTATAGTTTATCAAAGTAAAT
TATTAACTTTGAATGGTAAAAAATATTATTTTGATAATAACTCAAAAGCAGTTACCGGATGGCAAACTATTGATAGTAAA
AAATATTACTTTAATACTAACACTGCAG3'

APPENDIX II.

SEQ ID No.2

| | |
|---|---|
| Sequence Type: | Nucleotide |
| Sequence Length: | 1853 bp |
| Strandedness: | Double |
| Topology: | Linear |
| Molecule type: | Genomic DNA |
| Original source: | C. difficile |
| Immediate experimental source: | C. difficile by Hind III digestion |
| Features: | Not yet established. |
| Properties: | Not known. |

```
AAAATAGTAA AAAAGTAGTG ATAGCTGCTG  TAAACGGATT TGCTTTAGGT GGATGTGAAC 60
TTGCAATGGC ATGTGATATA AGAATTGCAT  CTGCTAAAGC TAAATTTGGT CAGCCAGAAG 120
TAACTCTTGG AATAACTCCA GGATATGGAG  GAACTCAAAG GCTTACAAGA TTGGTTGGAA 180
TGGCAAAAGC AAAACAATTA ATCTTTACAG  GTCAAGTTAT AAAAGCTGAT GAAGCTGAAA 240
AAATAGGGCT AGTAAATAGA GTCGTTGAGC  CAGACATTTT AATAGAAGAA GTTGAGAAAT 300
TAGCTAAGAT AATAGCTAAA AATGCTCAGC  TTGCAGTTAC ATACTCTAAA GAAGCAATAC 360
ACTTGGTGCT CAAACTGATA TAAATACTGG  AATAGATATA GAATCTAATT TATTTGGTCT 420
TTGTTTTTCA ACTAAACACC AAAAAGAAGG  AATGTCAGCT TTCGTTGAAA AGAGAGAAGC 480
TAAGTTTATA AAAGGGTAAA AATCTAATTA  AAAATATATT TGCTGTTAAC TAGAATAATT 540
TAAATAGTAT TTTAATCTAG TTAACATAAT  ATAAATACTA AAATAACAGG AGGTTATAAC 600
TATGAAATTA GCTGTAATAG GTAGTGGAAC  TATGGGAAGT GGTATTGTAC AAACTTTTGC 660
AAGTTGTGGA CATGATGTAT GTTTAAAGAG  TAGAACTCAA GGTGCTATAG ATAAATGTTT 720
AGCTTTATTA GATAAAAATT TAACTAAGTT  AGTTACTAAG GGAAAATGGA TGAAAGCTAC 780
AAAAGCAGAA ATATTAAGTC ATGTTAGTTC  AACTACTAAC TATGAAGATT TAAAAGATAT 840
GGATTTAATA ATAGAAGCAT CTGTAGAAGA  CATGAATATA AAGAAAGATG TTTTCAACTT 900
ACTAGATGAA TTATGTAAAG AAGATACTAT  CTTGGCAACA AATACTTCAT CATTATCTAT 960
AACAGAAATA GCTTCTTCTA CTAAGAGACC  AGATAAAGTT ATAGGAATGC ATTTCTTTAA 1020
TCCAGTTCCT ATGATGAAAT TAGTTGAAGT  TATAAGTGGT CAGTTAACAT CAAAAGTTAC 1080
TTTTGATACA GTATTTGAAT TACTTAAGAG  TATCAATAAA GTACCAGTAG ATGTATCTGA 1140
ATCTCCTGGA TTTGTAGTAA ATAGAATACT  TATACCTATG ATAAATGAAG CTGTTGGTAT 1200
ATATGCAGAT GGTGTTGCAA GTAAAGAAGA  AATAGATGAA GCTATGAAAT TAGGAGCAAA 1260
CCATCAATGG ACCACTAGCA TTAGGTGATT  TAATTGGATT AGATGTTGTT TTAGCTATAA 1320
TGAACGTTTT ATATACTGAG TTTGGAGATA  CTAAATATAG ACCTCATCAC TTTTAGCTAA 1380
AATGGTTAGA GCTAAGCAAT TAGGAAGAAA  AACTAAGATA GGATTCTATG ATTATAATAA 1440
ATAAGAATTT GGAATAAAAG TTTAAATAAA  ATTGTTTAAA AACAATTTCG ATATATGAAA 1500
AAATCTAATT TAATGGGGGT AATGATATGA  GAGAAGTAGT AATTGCCAGT GCAGCTAGAA 1560
CAGCAGTAGG AAGTTTTGGA GGAGCATTTA  AATCAGTTTC AGCGGTAGAG TTAGGGGTAA 1620
CAGCAGCTAA AGAAGCTATA AAAAGAGCTA  ACATAACTCC AGATATGATA GATGAATCTC 1680
TTTTTAGGGG GAGTACTTAC AGCAGGTCTT  GGACAAAATA TATAGCAAGA CAAATAGCAT 1740
TAGGAGCAGG AATACCAGTA GAAAAACCAG  CTATGACTAT AAATATACTT TGTGGTTCTG 1800
GATTAAGATC GTGGCGGGCA CTCCGAACGT  GAACGGGTCC GCGTCGACCT GCA        1853
```

CLAIMS:

1. An oligonucleotide showing substantially specific binding affinity towards C. difficile DNA, or RNA.

2. An oligonucleotide according to claim 1, showing substantially specific binding affinity towards the C. difficile A Toxin gene.

3. An oligonucleotide according to claim 2, comprising a sequence of at least 15 nucleotides the same as or complementary to any sequence of 15 or more nucleotides of the DNA sequence set forth herein and identified as the C. difficile A Toxin gene fragment.

4. An oligonucleotide according to claim 3, comprising a sequence of at least 15 nucleotides the same as or complementary to any sequence of 15 or more nucleotides at the 5' or 3' terminus of the C. difficile A toxin gene repeat sequence A, B, C or D.

5. An oligonucleotide according to any one of claims 1 to 4 in which the nucleotide sequence has from 15 to 50 units.

6. An oligonucleotide according to any one of claims 1 to 4 in which the nucleotide sequence is of 15 to 30 units.

7. An oligonucleotide according to claim 4, comprising a sequence of from 15 to 30 nucleotides in length and corresponding or complementary to a sequence of from 15 to 30 nucleotides at the 5' or 3' end of the C. difficile A toxin gene repeat sequence A or C.

8. The oligonucleotides:

BW69 5' GAAGCAGCTA CTGGATGGCA 3'
BW70 5' AGCAGTGTTA GTATTAAAGT 3'
BW65 5' ATAGGGGTGT TTAAAGGACC T 3'
BW66 5' AGCCTGACCT TCTATGTTAT 3'

9. An oligonucleotide according to claim 1, which is complementary to and hybridisable with the 1.8 kb HindIII restriction fragment of C. difficile DNA.

10. An oligonucleotide according to claim 9, comprising a sequence of at least 15 nucleotides the same as or complementary to any sequence of 15 or more nucleotide bases of the DNA sequence set forth herein and identified as the 1.8 kb HindIII restriction fragment of C. difficile DNA.

11. An oligonucleotide according to claim 9 or 10 in which the nucleotide sequence is from 15 to 30 units.

12. The oligonucleotides BW 71, 028, 029, 031, 032 or 037.

13. A DNA probe comprising an oligonucleotide according to any one of claims 1 to 12 labelled with a label permitting detection of the probe when hybridised to a complementary sequence of C. difficile DNA.

14. A DNA probe according to claim 13, wherein the label is radioactive, fluorescent or enzyme label attached to the oligonucleotide sequence.

15. A DNA probe comprising an oligonucleotide according to any one of claims 1 to 12 immobilised onto a solid support.

16. A method of detecting the presence of C. difficile in a sample, which comprises treating the sample to release the DNA or mRNA from any C. difficile present in the sample, and detecting that released DNA or mRNA for the presence of the C. difficile using an oligonucleotide according to any one of claims 1 to 12 or a DNA probe according to any one of claims 13 - 15.

17. A method according to claim 16, as applied to the detection of C. difficile in a sample from a patient.

18. A method according to claim 17, wherein said sample is a faecal sample.

19. A method according to claim 16, 17 or 18, wherein the detection process involves amplification of the released C. difficile DNA by a polymerase chain reaction (PCR) using a pair of oligonucleotides according to any one of claims 1 to 12.

20. A method according to claim 19 wherein the selected pair of primers is either BW69:BW70; or BW65:BW66 as identified in claim 8.

21. A diagnostic kit for the diagnosis of C. difficile infection in a patient comprising an oligonucleotide according to any one of claims 1 to 12 or a DNA probe according to claim 13, 14 or 15.

22. A diagnostic kit according to claim 21 which is a PCR kit comprising as primers for the PCR a pair of oligonucleotides according to any one of claims 1 to 12.

23. A diagnostic kit according to claim 22, comprising as primers for the PCR reaction the pair of oligonucleotides BW69:BW70 or BW65:BW66 as identified in claim 8.

FIG. 1

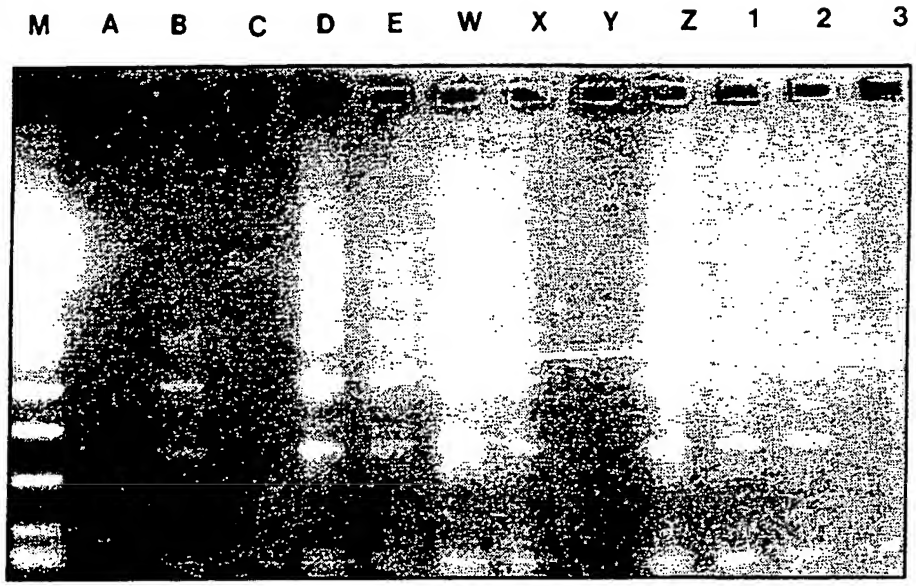


FIG. 2